THE SYNTHESIS AND ACTIVITIES OF ANTIBODIES

WILLIAM H. TALIAFERRO*
Department of Microbiology, University of Chicago

THIS evening I should like to consider the growing evidence that antibodies formed in a given species may differ widely in physicochemical properties and that these differences may be associated with marked differences in biological action. These findings, I believe, are of importance both to parasitologists interested in the action of antibodies on parasites and to other biologists using antibodies as experimental tools. The same material also forms an appropriate introduction to a discussion of the synthesis of antibody. The two subjects are so broad that I shall limit myself to a few illustrations, most of which are drawn from the work of the group associated with Dr. D. W. Talmage in the Department of Medicine of the University of Chicago and the group associated with Mrs. Taliaferro and me.†

Antibody has always been defined primarily in terms of its ability to combine with the antigen which induced its formation or with closely related substances and, secondarily, with its origin or increase following immunization. It was early recognized that most antigen-antibody reactions involve two stages: the first or primary rapid union of antigen and antibody and the second or visible stage, such as precipitation, lysis and biological neutralization. The so-called unitarian hypothesis gave a much needed simplification to the concept of antigen-antibody reactions. It led to the idea that the primary stage is fundamentally the same no matter what the secondary manifestation. Whether the latter is precipitation, lysis, etc., depends not on the nature of the antibody but on the physical state of the antigen, on environmental conditions, such as pH and salt concentration and, in some cases, on cooperative agents, such as phagocytes and complement (see Zinsser, 1931).

Most classic methods of measuring antibody have made use of the variable secondary stage. At the height of the reign of the unitarian hypothesis, this procedure offered little cause for concern since each

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† These include Dr. R. S. Farr of the University of Pittsburgh, Dr. B. N. Jaroslow of the Argonne National Laboratory, Dr. P. Stelos of Yale University, Dr. R. S. Weinrach of Northwestern University, Mrs. G. G. Freter of Jefferson Medical College in Philadelphia and Mr. P. D'Alesandro and Miss M. Lai of the University of Chicago.

antibody could presumably show any secondary stage provided the necessary conditions were satisfied. Evidence is accumulating, however, that antibodies produced in the same animal with the same specificity may vary in their ability to trigger or support the visible secondary stage. A well known early example of this is nonprecipitating antibody. Recently, increased attention has been paid to the primary union of antigen and antibody as measured by the antigen-combining capacity of antibody (review by Talmage, 1957a). Here, also, the physico-chemical properties of antibody are important.

I should like to illustrate some of the complexities of the antigen-antibody reactions by discussing certain aspects of the secondary immunological phenomena of precipitation, skin sensitization, lysis and ablastic action. These examples will bring out the difficulties in measuring the *total* amount of antibody in the serum and in definitively characterizing antibody. These are the phenomena with which you, as biologists, are particularly concerned. This point needs emphasis since biologists and clinicians will and should be most interested in the biological effects produced by the secondary stage of immunological reactions.

The secondary reaction of precipitation has become the central immunological reaction of the immunochemists. It is the simplest one since both reactants are in solution and do not need complex accessory agents. Even more important, exquisitely fine methods of weighing antibody have been developed, notably by Heidelberger and his coworkers. Nevertheless, the so-called quantitative precipitin test has drawbacks for biological workers. It does not necessarily measure the biological activity of antibody or the total amount of antibody since it selects antibody having special properties including a relatively high avidity and a low rate of dissociation.

An excellent example of the limited nature of the quantitative precipitin test is shown by Farr (1956) for the amount of antigen bound and precipitated by antibody in the P_{80} test and in the antibody-binding capacity (= ABC) test.* The P_{80} test of Talmage and Maurer (1953) determines the amount of precipitate at the point of 80% antigen precipitation and gives results closely similar to the quantitative precipitin test (cf. W. H. and L. G. Taliaferro, 1957). The antigen-binding capacity* is determined by the gamma ray counts of I^{131}-labeled BSA (bovine serum albumen) in precipitate when mixtures of varying amounts of I^{131}-labeled BSA and constant amounts of unlabeled antiBSA serum are mixed in 50% saturated ammonium sulfate. Under these conditions, the uncombined I^{131}-labeled BSA, being an albumin, remains in solution while the I^{131}-labeled BSA bound to antiBSA is precipitated along with

* In this test, the antigen has to be soluble in half saturated ammonium sulfate.

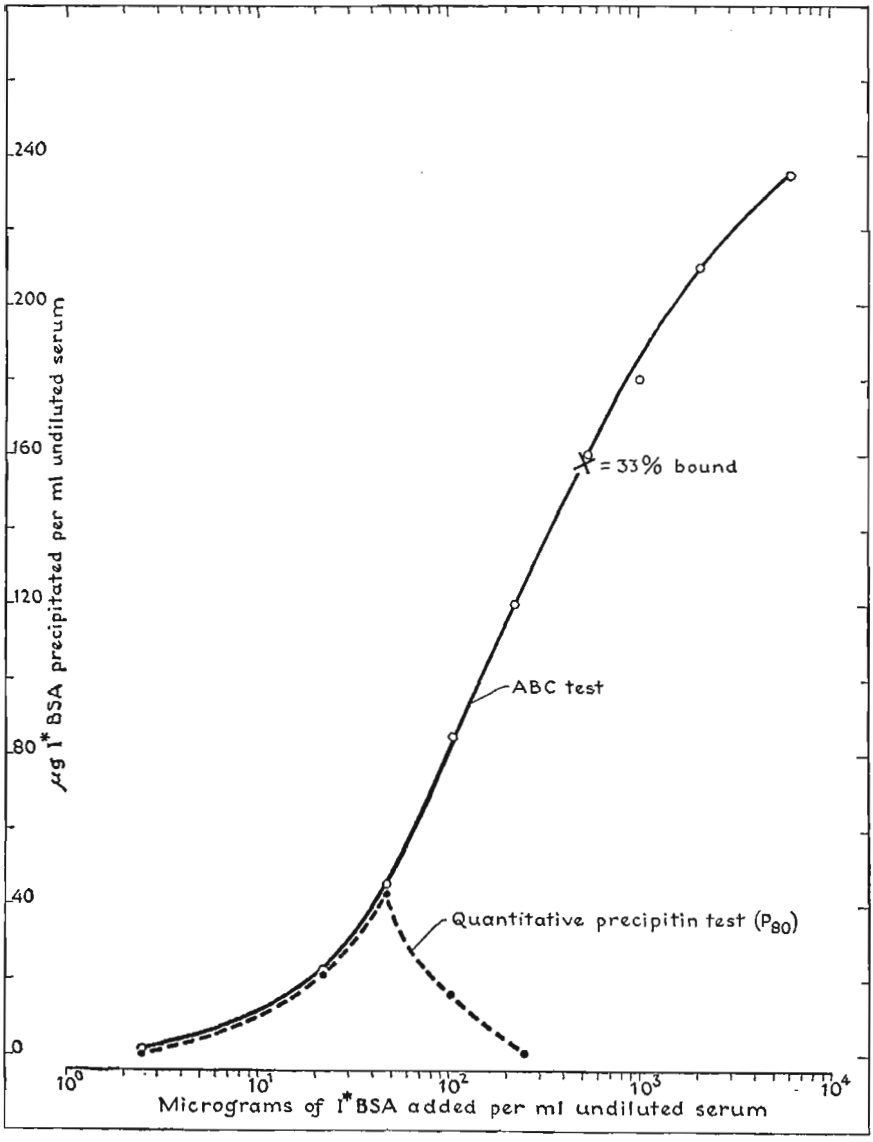


FIG. 1. The amount of antigen precipitated with antibody as measured by the quantitative precipitin (= P_{80}) test and the antibody-binding capacity (= ABC) test when increasing amounts of I^{131}-labeled BSA are added to a specific antiserum. (From unpublished data by Dr. R. S. Farr.) Note that per ml serum both tests registered practically the same amount of precipitable antigen up to 40 μg of I* BSA, but thereafter the tests registered markedly different amounts as additional antigen was added.

unlabeled antibody and other serum globulins. With the antiserum illustrated in figure 1, the maximum amount of antigen (40 μg. of BSA N) was precipitated in the P_{80} test by using 50 μg. of BSA nitrogen per ml. of antiBSA. Less antigen was precipitated when larger quantities of antigen were added. In marked contrast, the amount of antigen bound and precipitated increased as more and more antigen was added in the ABC test. At the highest point of the curve, 238 μg. of BSA nitrogen were precipitated by using 6000 μg. of BSA nitrogen per ml. of antiBSA. Farr further found that the ratio of antigen bound and precipitated in the two tests as well as the avidity vary widely among different serums.

Antibody acting as a precipitin has been of especial interest to parasitologists since the work of Blacklock, Gordon and Fine (1930) on fly larvae. Similar studies were made on various helminths by Sarles and Taliaferro (1936), Sarles (1938), Taliaferro and Sarles (1939 and 1942), and many subsequent workers. The reports indicate that precipitates may play a role in immunity to certain of the metazoan parasites. The formation of visible precipitates in and around fly larvae and worms is striking, but we may have gone too far in picturing the antibody effects in terms of this immune precipitate. As previously pointed out (Taliaferro, 1943), experiments on passive transfer are conclusive in showing that the prevention of feeding, stunting, immobilization, occasional death and expulsion of *Nippostrongylus muris* from the intestine are antibody effects, but the antibody need not necessarily act as a precipitin (i.e., be precipitating).

Kuhns and Pappenheimer (1952 a and b) and Kuhns (1954) have studied the biological antitoxic activity of nonprecipitating diphtheria toxin. They have clearly demonstrated the formation of two types of antitoxin in most persons immunized with diphtheria toxoid. The classical type is precipitating, is not skin-sensitizing since it leaves an injected site in about 100 minutes, and fixes complement in high titer. The nonprecipitating type is skin-sensitizing to toxin and toxoid since it remains at the site of injection for weeks. It fixes complement only slightly and loses its skin-sensitizing property after heating at 56°C for 4 hours. Both antibodies can passively sensitize guinea pigs to fatal anaphylactic shock. Briefly, therefore, the first type is a typical precipitating antibody, whereas the second one is indistinguishable from the heat labile, skin-sensitizing atopic antibody of asthma and hay fever. In spite of these differences, the nonprecipitating antibody is a highly effective antitoxin in neutralizing toxin in vivo. The nonprecipitating antibody is electrophoretically localized in the fast moving globulins (γ_1 or T fraction) and the precipitating antitoxin remains in the slow-moving globulins (γ_2 fraction). As my talk develops, we shall see that a number of biologically active antibodies belong to the fast moving or γ_1 globulins. The non-

precipitating antibody coprecipitates with precipitating antibody in the same way that nonprecipitating antibody frequently formed by rabbit does. Later, Kuhns (1955) found a second nonprecipitating antibody which diffuses rapidly from the site of inoculation and does not therefore sensitize the skin.

When one contemplates the implications of the various precipitating and nonprecipitating antibodies, the problem of accurate characterization is fascinating but certainly complicated. The situation would be somewhat simplified if each type of antitoxin, for example, had a different specificity and could neutralize the same toxin by combining with it at different sites or if the precipitating antitoxin combined with toxin or toxoid and the skin-sensitizing antitoxin combined with a tissue cell-toxin compound (review by Talmage et al, 1956). The possibility of an antitoxin to a tissue cell-toxin compound is suggested by the work of Ackroyd (1949) who demonstrated a sensitizing antibody to a sedormid (allylisopropylacetylurea)-platelet complex. Kuhns and Pappenheimer, however, believe that the ability of the precipitating and nonprecipitating antitoxins to neutralize the toxin is evidence of their identical specificity. Here, nevertheless, as in the later discussion of hemolysins, it is clear that there is no absolute method of determining specificity because two antibodies may combine with different sites on the antigen but may still be close enough to block each other partially or completely.

In many worm infections, a skin-sensitizing antibody is common. In 1928, Coventry and I found that 73% of 90 Honduraneans who were either currently infected or probably previously infected with ascaris gave the immediate wheal type of skin reaction after a scratch test with low concentrations of ascaris antigen, whereas the serums of 60% elicited antiascaris precipitins, 43% gave evidence of both antibodies and 11% gave neither. This scratch test with low concentrations of antigen is characteristic of the skin-sensitizing nonprecipitating antibody. The possible role of nonprecipitating and skin-sensitizing antibodies in worm infections seems to me one of the most intriguing problems in parasitic immunity.

Another pertinent illustration of the diversity of antibodies is found in the antisheep hemolysins formed by the rabbit which have held my interest for the past seven years. As far as is known they act as do other cytolysins including such antibodies as trypanolysins. Parenthetically, I should add that we left the trypanolysins and started work with hemolysins because hemoglobin released from the red cell by hemolysis can be accurately measured by modern photometric methods. By using these methods with a 50% endpoint, we possess one of the most delicate

and quantitative measures of the biological activity of antibodies so far devised.

Since the pioneer work of Forssman (1911) and Landsteiner and Prašek (1912), it has been known that immunization of rabbits with sheep red cells yields hemolysins with two specificities: one with Forssman and the other with isophile (i.e., sheep) specificity. The experimental studies of Dr. D. W. Talmage and his associates and our group indicate that within each specificity there are two hemolysins which have markedly different biological characteristics and activities. The difference in the half life of these four activities is shown in figure 2. The genesis of this

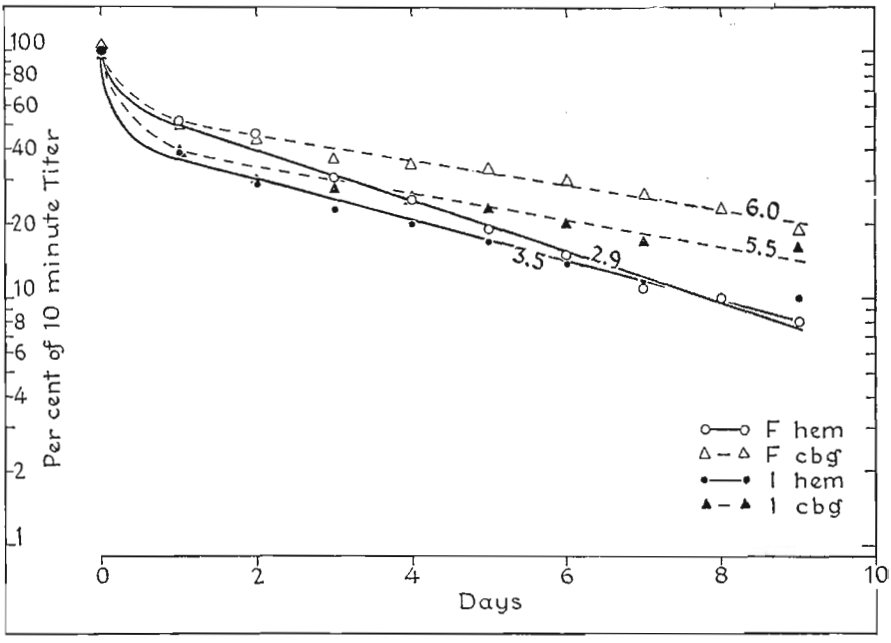


FIG. 2. The passive transfer into a normal rabbit of a rabbit antiserum containing four antibodies to sheep red cells. The anti-Forssman (F hem) and the isophile (I hem) hemolysins disappeared at faster rates (2.9 and 3.5 days) than the anti-Forssman (F cbg) and the isophile (I cbg) combining activities (6.0 and 5.5 days). From Taliaferro and Talmage, 1956.)

work was three-fold: the earlier work of Païc (1939) which indicated that the molecular weight of hemolysins was about 1,000,000 whereas that of most precipitins was about 160,000 (see Kabat and Mayer, 1948); my own interest in the biological half life of antibodies; and Dr. Talmage's interest in the relationship of the combining capacity and the hemolytic capacity of hemolysin in the presence of complement.

For simplicity I shall consider the two rabbit hemolysins of Forssman specificity as they are more easily studied because they predominate. One of these is a large molecule (ca 1,000,000 molecular weight) although small in total amount, is highly hemolytic, localizes during electrophoresis in the γ_1 (= T) or fast moving γ globulin fraction, and has a biological half life of 2.8 days. The other one is a smaller molecule (ca 165,000 molecular weight) although large in total amount, is slightly hemolytic, localizes during electrophoresis in the γ_2 or slow moving γ globulin and has a biological half life of 5.6 days. The foregoing characteristics are shown in table 1. During immunization, the large hemolytic antibody

TABLE 1

FORSSMAN ANTISHEEP RED CELL ANTIBODIES IN THE RABBIT*

Characteristics			Data from:*
Activity	Highly hemolytic	Weakly hemolytic	2
Molecular weight	1,000,000	165,000	1, 5
Rate of hemolysis	Varies as the square of the concentration	Varies as the 4th power of the concentration	6
Blood/tissue equilibration	$\frac{80}{20}$	$\frac{50}{50}$	3
Electrophoretic mobility	γ_1 (T fraction)	γ_2	1, 2
Peak titer	Early	Late	4
Half life	2.81 ± 0.12 days	5.56 ± 0.17 days	3

* The data were obtained from the following six references: (1) Stelos (1956); (2) Stelos and Talmage (1957); (3) Taliaferro and Talmage (1956); (4) Talmage et al. (1956a); (5) Talmage et al. (1956b); (6) Weinrach and Talmage (1958).

is formed first and reaches a high titer by the 5th day. The smaller slightly hemolytic one appears later and reaches a peak on about the 25th day at a time when the titer of the large one is decreasing. These differences are shown in figure 3. Although hemolytic units (measured by the amount of hemoglobin released) and combining units (measured by the ability of an antiserum to block the uptake of a standardized I^{131}-labeled antibody on red cells) actually include both antibodies, the tests can be used to differentiate the two because of their markedly different characteristics. Thus, the small antibody is so poorly hemolytic that it adds very little to the total amount of hemolysis. Conversely, the large antibody is proportionately so small in amount that it adds very little to the total combining capacity. Therefore, hemolysin titer can be ascribed to the large molecule and combining activity to the small one. The hemolytic efficiency is the hemolytic titer divided by the combining titer. As shown in figure 3, this efficiency declines as the combining titer

increases. The two types of hemolysin have also been found by Stelos (1958) with a bovine isophile specificity after immunizing rabbits with bovine cells.

The work of Weinrach and Talmage (1958) gives a probable explanation of the difference in hemolytic efficiency of the two types of antired-

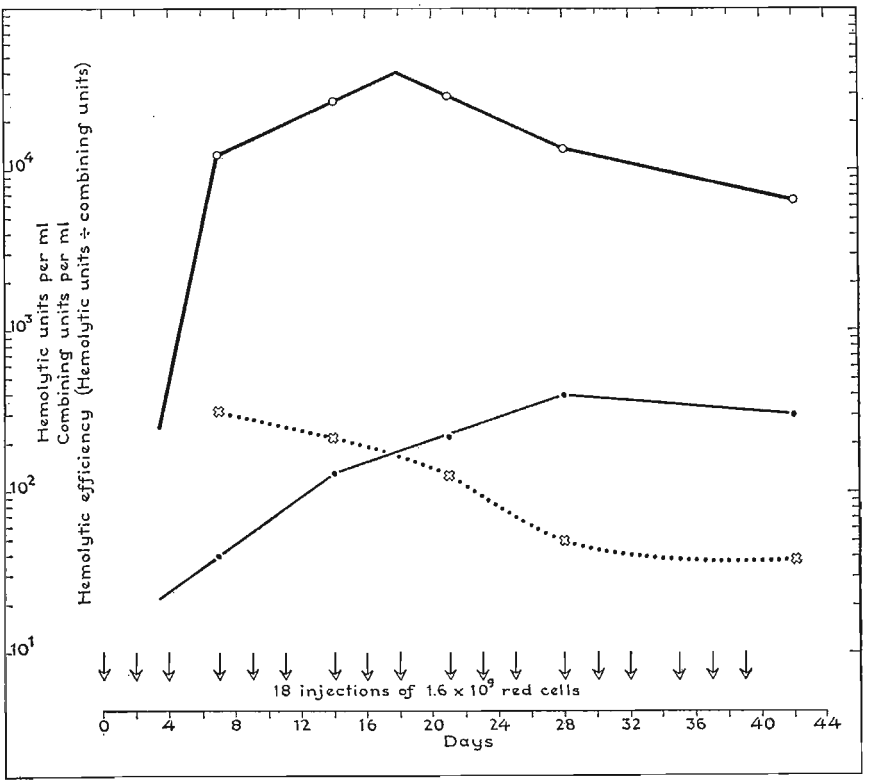


FIG. 3. The mean course of hemolysin (heavy solid line) and combining activity (fine solid line) together with the resultant hemolytic efficiency (dotted line) in the antiserums of 6 rabbits repeatedly injected intravenously with sheep red cells. These activities were of the anti-Forssman type. (Modified from Talmage et al, 1956a.)

cell antibody. For example, the rate of hemolysis varies with the square of the concentration of the larger molecule and as the fourth power of the smaller one. The results indicate that a two-molecule complex is necessary to produce a single hemolytic injury by the large antibody, whereas a four molecule complex is necessary to produce such an injury by the small one. There is, however, an anomaly in this comparison of hemolytic capacity. The comparison holds when titers are determined by the highest dilution that will give a specific effect because

there are too few molecules of either antibody to saturate the red cell. Under biological conditions, however, when a red cell is injected or a parasite enters the blood, there are frequently sufficient molecules of each antibody in the undiluted plasma to saturate the antigen and cause lysis almost instantaneously.

Two questions immediately arise. Are these two antibodies of identical or only of closely related immunological specificity? If they are of identical specificity, is the formation of two antibodies with such different biological activities characteristic of certain antigens (such as red cells) or of all antigens in the rabbit? Unfortunately, we can answer neither of these questions categorically. The two antibodies must be similar, if not identical, in specificity, because they cross-block each other, i.e., "saturation" of a red cell with one type prevents adsorption of the other, and it is possible for an avid small antibody to combine with so many closely adjacent sites on a cell that the action of the hemolytic antibody will be greatly inhibited. Whether every antigen injected into rabbits produces antibodies of the two molecular sizes cannot be answered so far, as we have at present no test for any antibody of the large molecular type except as a hemolysin.

The difference in molecular weight of the two hemolysins makes it possible to concentrate the hemolytic activity by centrifugation. Talmage et al (1956b) found a 4-fold concentration of the hemolytic activity as contrasted to a 2-fold concentration of combining capacity in the bottom third of a serum which was centrifuged for 3 hours in an average gravitational field of 105,000 $\times$ g. Stelos and Talmage (1957) used starch block electrophoresis to make a much sharper separation of the two types. As is clearly shown in figure 4, most of the hemolytic activity is in the γ_1 or T fraction, whereas most of the combining capacity is in the γ_2 fraction. This difference is strikingly shown by the hemolytic efficiency, i.e., hemolysin/combining ratio (= H/C) which is 834 in the γ_1 and only 9 in the γ_2 fraction.

The mechanism of hemolysis is a fascinating and complex process. It is generally assumed that the hemolytic antibody combines with the red cell and in the presence of complement induces the lytic injury. The work of Mayer et al (1948) indicates that, during this process, complement is destroyed whereas antibody dissociates and can initiate a series of lytic injuries on the same or other red cells provided sufficient complement is present. The degree of avidity of hemolysin as expressed in the rate of dissociation or movement from site to site on the same or different red cells is of great importance in lysis. Weinrach and Talmage (1958) suggest that there is an optimal avidity which gives maximum hemolysis. Below the optimum, the antibody would be relatively inefficient because it would frequently dissociate before lytic injury would be completed

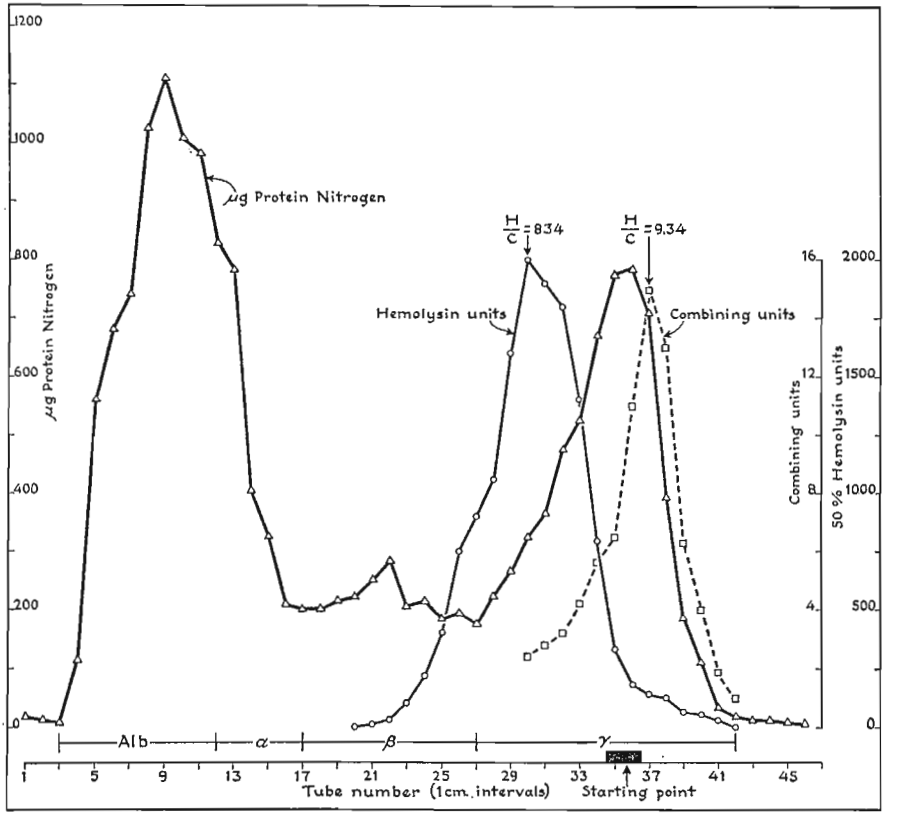


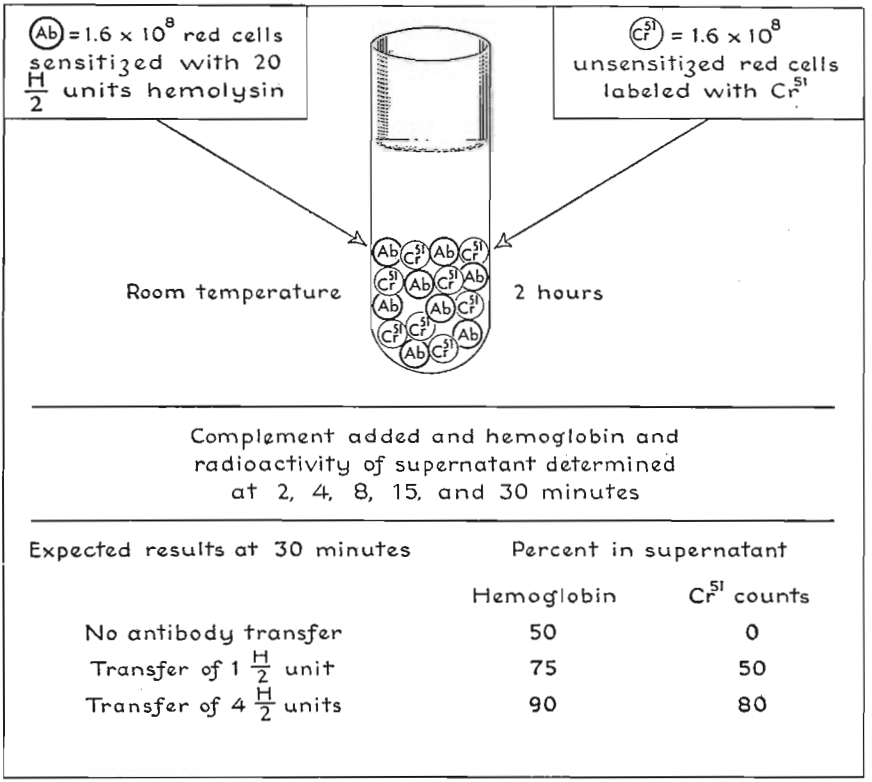
FIG. 4. The electrophoretic distribution of protein nitrogen and of Forssman hemolysin and combining activity in an antiserum from a rabbit 2 days after the last of 8 intravenous injections of 1.6×10^9 sheep red cells per kg during a 2-week period. (From Stelos and Talmage, 1957.) Peaks in hemolytic titer and combining titer were reached in such different fractions that the ratio of the two titers, i.e., the hemolytic efficiency (H/C), at the two peaks differed by a factor of over 90.

and, above the optimum, it would dissociate so slowly that it would produce only a few lytic injuries.

Avidity was first used to explain the difference in capacity of the diphtheria antitoxin to precipitate with toxin in vitro and to neutralize toxin in vivo (cf. Barr, 1951). It has been related to the rate of the secondary reactions, such as neutralization, precipitation and agglutination, and also to the equilibrium between antigen and antibody in the primary reaction. This equilibrium is probably modified by factors ranging from immunological valence, goodness of fit of the combining groups and solubility of the antigen-antibody complex. The avidity of antibody increases with time after a single injection of antigen and is especially high during an anamnestic response.

Bowman et al (1951) first demonstrated the transfer of hemolysin from both stromata and red cells to other red cells. It is generally assumed that intersite transfer on the same cell is more important than intercellular transfer but the latter probably gives a relative measure of the former. We (Taliaferro et al, 1958) have, therefore, developed a quantitative measure of intercellular transfer based on the work of Weinrach et al (1958). Rachromate (Na_2 $Cr^{51}O_4$) was used to label red cells because Cr^{51} specifically labels hemoglobin and thus makes it possible to determine the hemoglobin derived from chromium-labeled cells. The actual measurement of intercellular hemolysin transfer was carried out as follows: Washed normal sheep red cells (1.6×10^8) are sensitized with twenty 50% units of a rabbit Forssman hemolysin. The sensitized unlabeled cells are put in contact with an equal number of unsensitized Cr^{51}-labeled cells for two hours at room temperature. During this interval, intercellular transfer of antibody can take place from sensitized unlabeled to unsensitized Cr^{51}-labeled cells. The mixture is then incubated at 37°C for 30 minutes with excess complement, and hemolysis is stopped abruptly with citrate. In the supernatant, total hemoglobin is determined photometrically and Cr^{51}-labeled hemoglobin is determined by gamma counts. The procedure and three possible results are given in figure 5. As shown in the first line of results, no antibody was transferred because 50% of the total cells were hemolysed, and no Cr^{51} occurred in the hemoglobin of the supernatant. Since the sensitized cells were not saturated with antibody, hemolysin which left them could attach itself to sensitized or to Cr^{51}-labeled cells at random. Thus, in the second line of possible results in figure 5, one 50% hemolytic unit was transferred because 75% of the total cells were hemolysed and 50% of the total Cr^{51} available was in the hemoglobin of the supernatant. In a study of various serums, we have found that 5 or more 50% hemolytic units transfer in nonavid serums from normal rabbits (which contain low-titered natural antibody) or from rabbits within 4 days after a single injection of heated sheep red cell stromata, whereas frequently less than one half of a hemolytic unit transfers in avid serums at peak titer during initial and anamnestic responses and for several weeks thereafter. The great increase in avidity which takes place shortly after 4 days following a single injection of antigen must chiefly involve the large hemolytic antibody since the small antibody appears later as shown in figure 3. It is thus evident that smaller amounts of nonavid serums are needed to produce a given amount of hemolysis under the conditions of our titration than are required with avid serums.

My coworker, Mr. Philip D'Alesandro (unpublished work) has found that both the ablastin which inhibits reproduction early in infections with



Expected results at 30 minutes	Percent in supernatant	
	Hemoglobin	Cr^{51} counts
No antibody transfer	50	0
Transfer of 1 $\frac{H}{2}$ unit	75	50
Transfer of 4 $\frac{H}{2}$ units	90	80

FIG. 5. Schematic representation of the intercellular transfer of hemolysin (in terms of 50% units) from unlabeled sensitized to Cr^{51}-labeled unsensitized sheep red cells during 2 hours in vitro contact at 22°C. (From unpublished data by W. H. and L. G. Taliaferro and Pizzi.)

Trypanosoma lewisi and the lysin which terminates the infection (Taliaferro, 1924, 1932 and 1948) have about the same electrophoretic mobility and localize in the γ_1 or T fraction. The lytic activity is associated with a large molecule (S_{20} = 16S) similar to the hemolysin of high molecular weight, whereas preliminary work indicates that ablastic activity is associated with a small molecule (S_{20} = 6S) similar to the small hemolysin.

Recently, Terry (1957) found an innate principle in the serum of cotton rats which agglutinates and lyses *T. vivax* in vitro and appears to be responsible for the complete resistance of this species to infection with the trypanosome. Like the acquired lysin to *T. lewisi*, this innate lysin to *T. vivax* is found in the β and fast moving γ globulins (probably what we term the γ_1 or T fraction).

The preceding few examples show the diversity of antibodies in physico-chemical characteristics, their avidity in the first stage antigen-antibody union and their ability to induce and support the various secondary reactions. They also show that we have no method of measuring *total* antibody chiefly because we have no inclusive definition of antibody. In spite of this drawback, we have excellent *relative* methods of measuring some antibodies, i.e., the quantitative precipitin methods for avid precipitating antibodies, the antigen-binding capacity test of Farr (1956) for precipitating and nonprecipitating antibodies, the combining capacity test of Talmage and Freter (1956) for antired-cell antibodies and the 50% end point test (cf. W. H. and L. G. Taliaferro, 1950 and 1956) for hemolysins.

The lack of methods for measuring total antibody has not hindered the accumulation of considerable data on the synthesis of the antibody molecule. Most of these data parallel the data on the synthesis of other mammalian proteins and the induced enzyme proteins of bacteria.

There seems little doubt that all or part of the antigen molecule induces the body to start or to increase the production of antibody. Several theories have been advanced as to how this induction can take place (see Burnet and Fenner, 1949, Burnet, 1956 and 1958, Jerne, 1955 and 1956, and Talmage, 1957b), but experimental evidence is lacking. There is no agreement as to whether antibody is the increased production of a normal globulin which happens to react with the antigen, or a slightly modified normal globulin, or an entirely new globulin.

Recently, Jaroslow and I have probably found some of the materials which must be present to initiate antibody formation. We assumed from X-ray studies that irradiation destroys something necessary for the initiation (possibly the induction) of the immune process. Thus, it had been known for some time that early stages of the immune process are X-ray sensitive, whereas later stages are relatively X-ray resistant (review by W. H. and L. G. Taliaferro, 1951a, and Kohn, 1951). Dixon et al (1952a) and Taliaferro et al (1952) showed that the early radiosensitive period could only be a few hours in duration and might be much shorter. Later, we (W. H. and L. G. Taliaferro, 1954) found that the power to form hemolysin decreases with an apparent half life of about 2 hours as tested in groups of rabbits injected with 2×10^7 sheep red cells before and after total body administration of 500 r. The data are shown in figure 6. In 1955 and 1956, it had been further shown by the Harrises and their associates that antibody formation can be restored in X-rayed (425 r) rabbits when the antigen is injected with lymph node cells either separately or after incubation together. In a somewhat similar manner, Jaroslow and I (1956) found that the hemolysin-producing capacity is re-

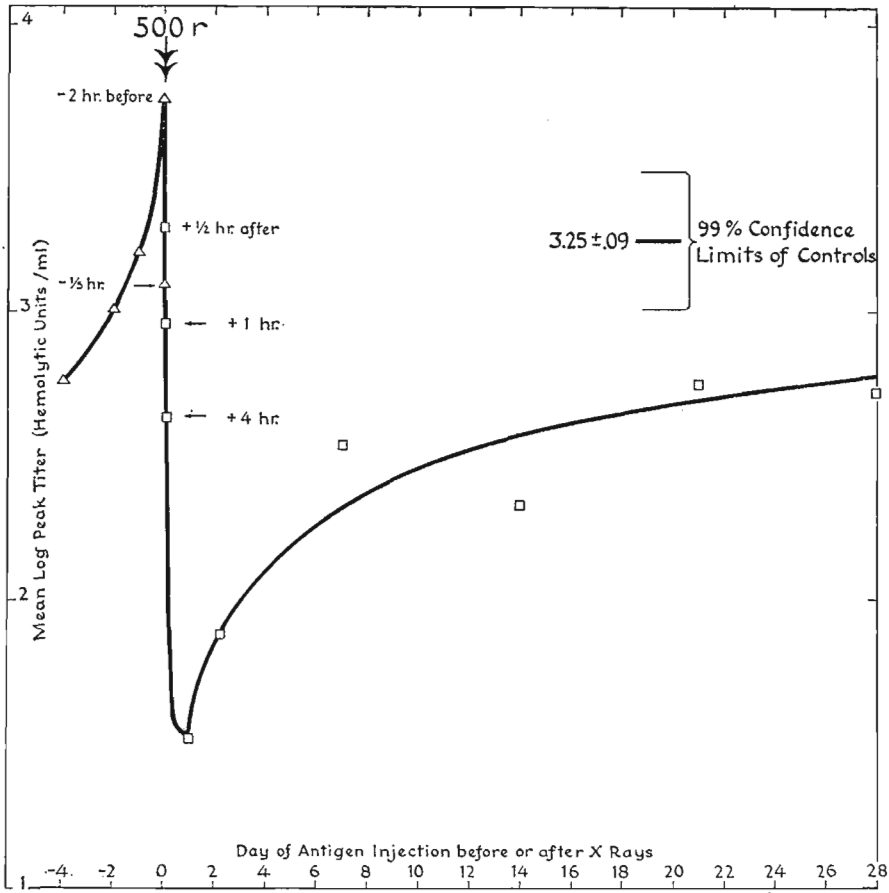


FIG. 6. Mean log peak hemolysin titer in the antiserums from 14 groups of rabbits as related to the time of the intravenous injection of 2×10^7 sheep red cells per kg rabbit 4 days before through 28 days after total body irradiation. (Redrawn from W. H. and L. G. Taliaferro, 1954.) Note the precipitous drop in peak titer when antigen was given from 2 hours before to 4 hours after irradiation.

stored to a small but significant extent when sheep red cells are mixed and injected with a mince of normal rabbit spleen into rabbits irradiated one day previously with 400 r. We soon found, however, that the restoration is not mediated by specific cells and does not require intact cells. Mince of mouse spleen is as effective as rabbit spleen, and HeLa cells are even more effective. Furthermore, certain tissue and cell extracts are satisfactory. Then yeast autolysate was found to give essentially complete restoration. Minces of rabbit kidney or of rabbit muscle give essentially negative results. The experimental data from treated groups

of X-rayed rabbits with controls are shown in figure 7. Unpublished work indicates that restoration of the hemolysin-forming capacity can be effected in irradiated rabbits to the same degree as splenic mince by products of the action of the specific nuclease on ribonucleic acid or on deoxyribonucleic acid. Neither nucleic acid has been found active. The only chemically defined nucleic acid derivative we have found to equal

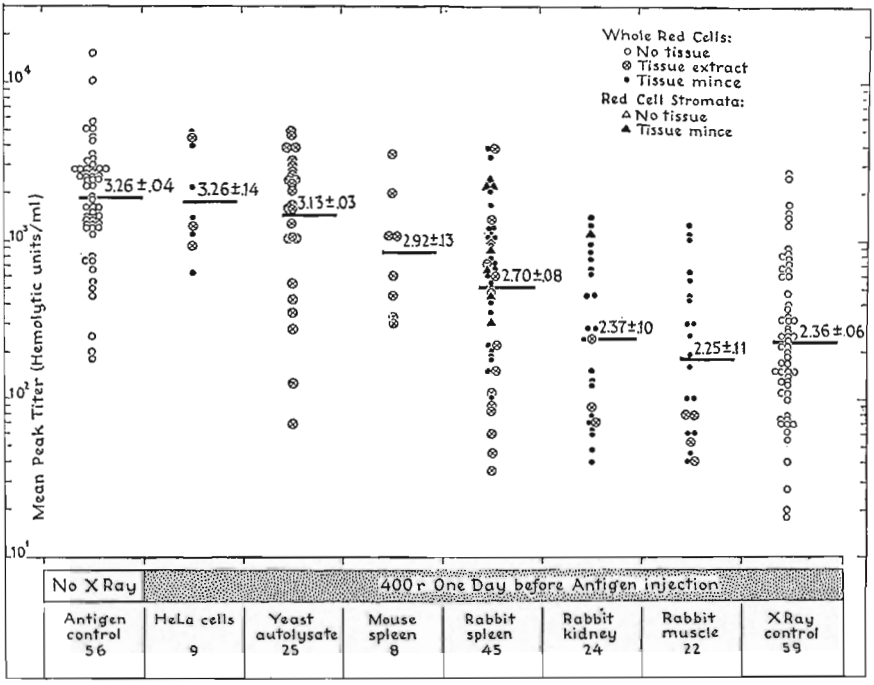


FIG. 7. Individual and mean log peak hemolysin titers in the antiserums from 6 groups of rabbits receiving total body irradiation before the intravenous injection of sheep red cells or heated stromata and various additional substances. Data are also included from nonirradiated and irradiated control groups receiving antigen alone. (Modified from Jaroslow and Taliaferro, 1956, with additional data.)

the activity of splenic mince is kinetin (= 6-aminofurfurylpurine), a derivative of deoxyribonucleic acid. We have no explanation of how the restorative materials act, but it seems likely that the yeast autolysate contains kinetin or other nucleic acid derivatives together wih a further substance which mediates some interaction between antigen and nucleic acid derivative. The whole process is probably a part of the nucleic acid control of protein synthesis.

Some of our recent experiments also indicate that these restorative substances when injected with sheep red cells into nonirradiated rabbits

lead to above normal amounts of hemolysin. At present, we interpret these results to indicate that necessary compounds are in marginal amounts at critical sites after irradiation and are below an optimal concentration in normal animals.

There is a difference of opinion among immunologists as to whether or not antibody formation occurs in the complete absence of antigenic stimulation. Our own work on hemolysins indicates that it cannot. Thus, figure 8 shows the daily output of hemolysin, corrected for metabolic

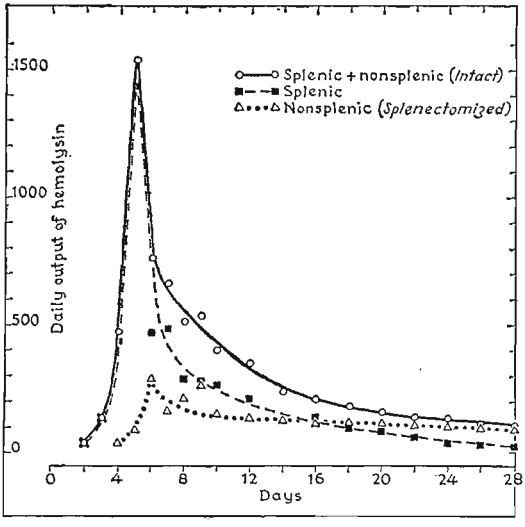


FIG. 8. Daily output of splenic and nonsplenic hemolysin calculated from hemolysin curves in normal and in splenectomized rabbits after a single intravenous injection of 1.6×10^9 sheep red cells per kg. (From Taliaferro, 1955.) Note that production by the spleen reached a high peak rapidly and then decreased rapidly while that by nonsplenic sources rose later to a lower peak and decreased slowly.

decay, by the spleen and by nonsplenic sources in the rabbit following a single intravenous injection of antigen. In this figure, nonsplenic output is considered to be that of splenectomized animals, and splenic output is assumed to be the difference between the daily output in intact and in splenectomized animals. The daily output of splenic hemolysin rises rapidly and falls off sharply (half life of about 2 days). The sharp decline in production is connected with the absence of antigenic stimulation since a second injection of antigen produces a second cycle of splenic activity comparable to the first (W. H. and L. G. Taliaferro, 1952). Nonsplenic hemolysin reaches a lower peak later and decreases more slowly. Actually, antibody production by nonsplenic sources for 3 weeks about equals that of the spleen for 1 week. The correlation between rate and length of antibody synthesis suggests that antigen is exhausted during the process of antibody formation.

If the antigen as a whole or in part is the actual inducer, the situation may be somewhat analogous to that governing the inducible enzymes of

bacteria. A few molecules of these enzymes are formed by the bacteria without the inducer, but a greatly accelerated production follows in the presence of the inducer. Furthermore, except in special cases, the inducer must be present for continued accelerated production.

During a long-continued regimen of antigenic stimulation, antibody decreases. This decrease may in part be associated with the absorption of antibody by antigen. In some systems, there may be a paralysis of the immune mechanism, as described by Felton (1949). Conversely, antigen may remain in the body but for various reasons may not effectively stimulate antibody formation. In our own work (1951b and 1952) involving injections of red cells into rabbits for two or three months, the spleen forms more hemolysin than after a single injection, but its output falls off in much the same way as after a single injection. Further experiments indicate that the decline in serum antibody is not caused by absorption or by a paralysis of the immune mechanism. It is a true refractory stage which occurs during antigenic stimulation and is in part controlled by the avidity and amount of circulating antibody. Thus, Talmage, Freter and Thomson (1956) found that the hemolysin response is greatly reduced when a mixture of red cells and serum from a refractory rabbit is injected into nonimmunized rabbits (see also Barr et al, 1950, and Mason et al, 1955).

Serum antibodies as well as other serum proteins, once formed, undergo metabolic degradation into their constituent amino acids which are thus added to the amino acid pool and become available for the synthesis of other proteins. This process is frequently termed recycling. In general, the rate of metabolic decay is faster in small animals such as the mouse with high metabolic rates than in larger animals such as the rabbit with low metabolic rates. Metabolic rate is only one factor, however, because antibodies decay more slowly in children with their higher metabolic rate than in adults (Dixon et al, 1952b). Even more striking is the fact already mentioned that two antibodies with closely related, if not identical, immunological specificity, may be formed in the same animal and decay at different rates (table 1 and fig. 2). In spite of the many factors influencing the rate of decay, the rate for a given antibody in a given species is remarkably constant under laboratory conditions as is illustrated by the small standard errors shown in table 1. Previous immunization and, hence, the presence of the synthesizing mechanism does not modify the rate of metabolic decay (Humphrey and McFarlane, 1954, and Taliaferro and Talmage, 1956). Moreover, antibodies and other serum proteins have remarkably fast metabolic turnovers in mammals (cf. Whipple 1956). At the other extreme, collagen has an extremely slow turnover (Neuberger et al, 1951, and Thompson and Ballou, 1956).

Although antibody is constantly being degraded into its constituent amino acids, there is no exchange between its amino acids and certain of its atoms and homologous amino acids and atoms in other compounds of the body *as long as the antibody retains its immunological reactivity.* This conclusion was first reached by Heidelberger and his associates (1942). It is best shown by passively transferring isotopically labeled antibody to an unlabeled recipient (see Gros et al, 1952b, Dubert et al, 1953, Humphrey and McFarlane, 1954, and the review by Taliaferro, 1957). Figure 9 illustrates an experiment of this type with S^{35}-labeled antiserum (Taliaferro and Talmage, 1955). The specific radioactivity of antibody in the labeled donor was 40.6 counts per minute per μg. antibody nitrogen. When introduced into the recipient, which was unimmunized and therefore neither contained nor made unlabeled antibody to dilute the radioactivity, the S^{35}-labeled antibody retained its original radioactivity for 9 days. During this time, the antibody was constantly decaying and only about 25% of the amount originally injected was pres-

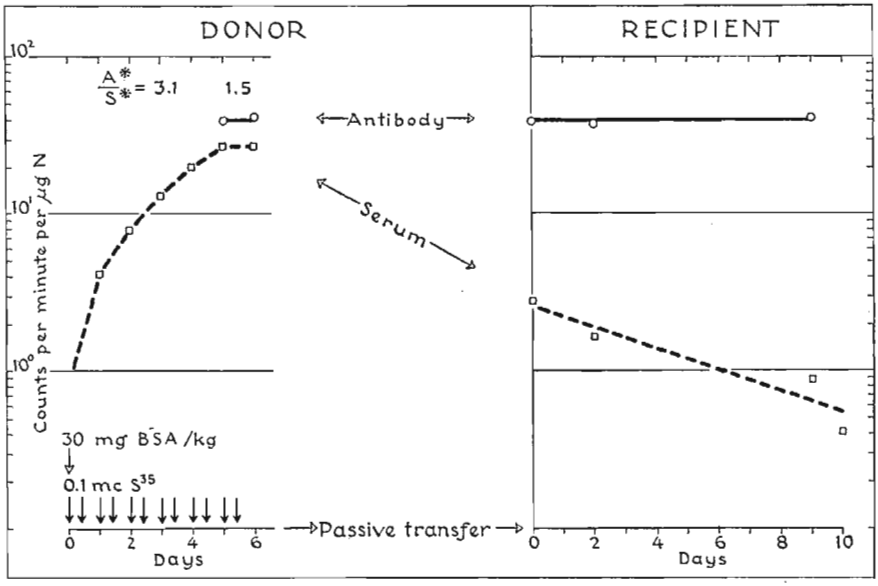


FIG. 9. The passive transfer of an antiserum containing S^{35}-labeled antibovine-serum-albumin and S^{35}-labeled serum proteins to a normal recipient. The S^{35} counts per μg antibody N (solid lines) remained constant in the donor and recipient whenever antibody determinations could be made while the S^{35} counts per μg serum protein N (dash lines) increased in the donor ($A^\circ/S^\circ = 3.1$ on day 3 and 1.5 on day 5 and 6) and decreased in the recipient. Precipitin titer is not shown in the figure. It rose rapidly beginning on day 3 in the donor and declined in the recipient. (From Taliaferro and Talmage, 1955.)

ent on the ninth day. It is obvious, therefore, that no atomic sulfur or nonlabeled sulfur-containing amino acids replaced a significant amount of the istopically labeled sulfur in the antibody. The stability of the antibody molecule is important because upon it are based some of the methods used in the study of antibody synthesis. The recipient, on the other hand, contains and makes unlabeled nonantibody serum proteins which immediately and progressively dilute the S^{35}-labeled nonantibody serum proteins as shown in figure 9.

Irrespective of whether antibody formation is the result of the increased formation of selected normal globulins or the synthesis of a new globulin, it is synthesized de novo in that the antibody molecule is formed directly from the amino acids of the amino acid pool (Green and Anker, 1954, and review by Taliaferro, 1957). It is not formed directly from preexisting globulins or other proteins (Gros et al, 1952a, Dubert et al, 1953, and Askonas et al, 1956). In this respect, it is similar to the enzyme proteins of bacteria (Rotman and Spiegelman, 1954, Hogness et al, 1955, and Monod, 1956). The actual time of synthesis is probably very short. Long-lived amino acid-containing precursors during synthesis were postulated earlier for antibodies and other proteins, but probably do not occur.

Long-lived precursors of antibodies would seem most likely to occur during the induction period of the immune process, i.e., after the introduction of the antigen but before the appearance of serum antibody. In fact, they seemed to account for the findings of Green and Anker in 1954. These authors administered three different isotopically labeled amino acids during the secondary response to ovalbumin—two during the induction period and one during the rise of serum antibody. The relative concentration of isotopes in antibody removed on the 5th day led these authors to conclude that 31% of the amino acids in the antibody were withdrawn from the amino acid pool during the induction period and the remainder from the pool during the rise of antibody in the serum.

Different results were obtained by Taliaferro and Talmage (1955), Stravitsky (1956 and 1957) and W. H. and L. G. Taliaferro (1957). As shown in figure 10, we divided the secondary immune response to bovine serum albumin between two rabbits. The induction period was passed in a donor which received one intravenous reinjection of BSA and twice daily doses of S^{35}-labeled amino acids. The spleen of the donor before precipitins appeared was then removed, minced and injected intravenously into an unimmunized unlabeled recipient. Antibody probably began to be made immediately in the recipient, but was labeled to a very small percent of that of the donor antibody. Parenthetically, it should be noted that we can be sure that antigen in the transferred spleen was

not responsible for the antibody made in the recipient because the antibody was all synthesized before 6 days which is the earliest time antibody appears after an initial injection of antigen (cf. Knox and Endicott, 1950).

The reciprocal relationship between the fall of specific radioactivity and the increase in antibody in the recipient (fig. 10) indicates that the recipient made small amounts of highly labeled antibody at first from the S^{35}-labeled materials carried over in the spleen from the donor and then made unlabeled antibody from the recipient's own amino

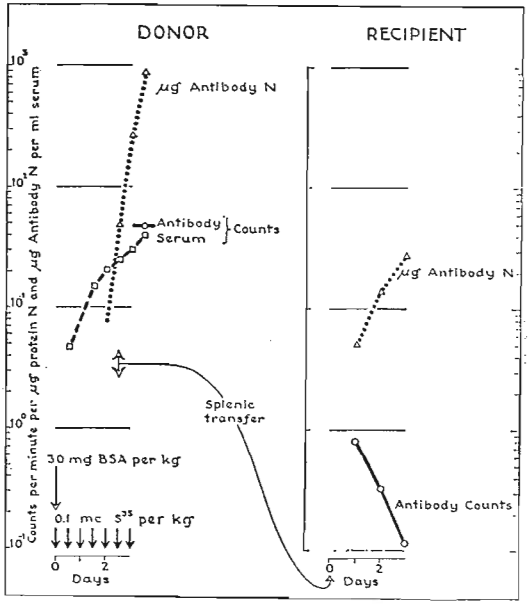


FIG. 10. The transfer to a normal recipient of spleen mince from a donor, 2.5 days after an intravenous injection of bovine serum albumin (= BSA) and after 5 intravenous injections of S^{35}-labeled amino acids. (Data from W. H. and L. G. Taliaferro, 1957.) In the recipient, precipitins (dotted lines) increased from day 1 (first determination) through day 3 while the S^{35} counts per μg antibody N (solid lines) were markedly lower than those in the donor on day 1 and decreased through day 3. The S^{35} counts per μg serum protein N (dash line) increased in the donor as in figure 9.

acids which progressively diluted the S^{35}-labeled antibody. On the third day, the S^{35}-labeling of the recipient antibody was only 0.02% of the specific radioactivity of the donor antibody. Thus, not more than 0.02% of the amino acids of the recipient antibody could have been drawn from the amino acid pool of the donor. We (Taliaferro and Talmage, 1955) accounted for Green and Anker's results by assuming that isotopic-labeled complement and coprecipitating antibody were carried down and included with the precipitating antibody removed on the 5th day. Dixon et al (1956) suggested that their results may have been due to recycling. Both factors were probably operative.

The transit time is the period elapsing between the administration of isotopically labeled amino acids and their presence in antibody in sufficient concentration to be demonstrated by laboratory procedures. During

the rapid rise of the secondary response to bovine serum albumin in the rabbit, Mrs. Taliaferro and I (1957) have found this interval to be not less than 20 minutes and not more than 40 minutes. Figure 11 shows the beginning and subsequent rapid rise in the incorporation of S^{35} amino acids into antibody. It seems significant that our determination of the

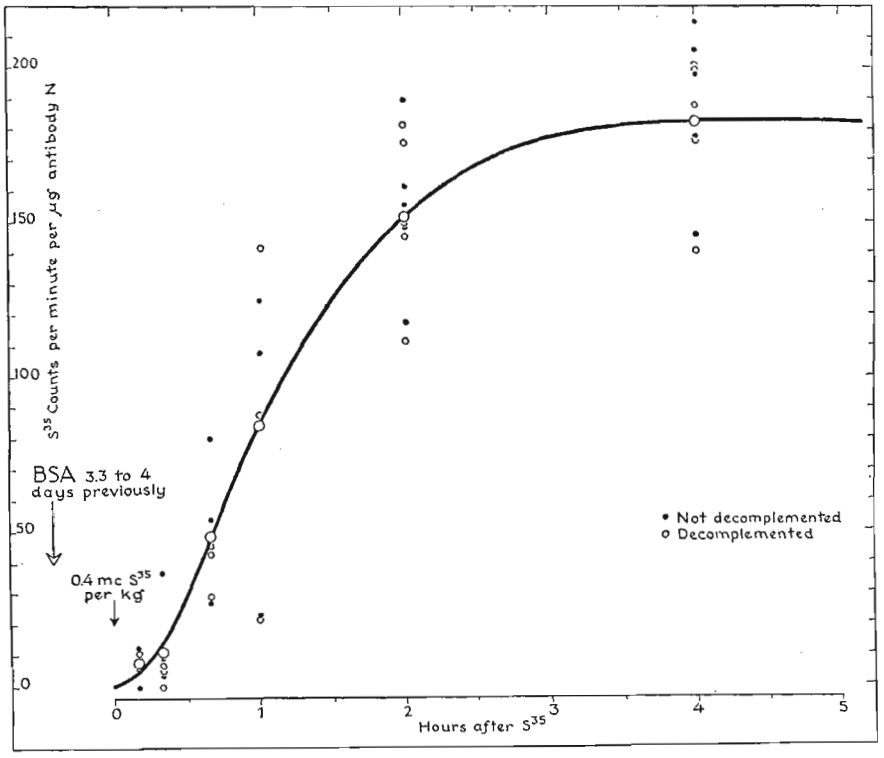


FIG. 11. The incorporation of S^{35}-labeled amino acids into antibovine-serum-albumin (= antiBSA) in rabbits during a rapid secondary rise of precipitins as shown by S^{35} counts of antibody for 5 hours after one intravenous injection of S^{35}-labeled amino acids. The large circles are mean values of the smaller circles and dots which were obtained with and without decomplementing the antiserums before testing. (From W. H. and L. G. Taliaferro, 1957.)

transit time in vivo is almost identical with that obtained for the incorporation of C^{14} in vitro by Askonas and White (1956). Thus, there seem to be no important factors holding up antibody in vivo that do not act in vitro. This length of time also agrees excellently with the approximate 30-minute transit time reported by Peters (1953) for the formation in vitro of chicken albumin, by Green and Anker (1955) for the formation of serum proteins in vivo in the rabbit, by Junqueira et al (1955) for the

formation of pancreatic proteins in vivo in the rat and by Askonas et al (1956) for the formation of γ globulin in vivo in the rabbit.

The period of not more than 40 minutes represents the maximum time necessary to assemble amino acids into rabbit antibody. It thus includes the maximal length of life of intermediates as well as the time necessary for amino acids to penetrate the antibody-forming cells and for antibody to leave the cells and reach the serum. The actual time of synthesis and, therefore, the maximum length of life of intermediates must be less than 40 minutes and may be only a few minutes as indicated for the induced bacterial enzymes (Hogness et al, 1955).

The absence of long-lived amino acid precursors and the short transit time indicate that, for antigens injected intravenously into the rabbit, serum titers suitably corrected for metabolic decay give a good approximation of the rate of antibody synthesis. Indirect evidence that this is true is given by the similarity of the results of Dixon et al (1956) on the rate of synthesis of antibovine serum albumin in the rabbit as measured by isotopic methods and our results with hemolysins using the changes in serum titer corrected for metabolic decay. Dixon and his associates reported that the rate of synthesis after peak titer fell off rapidly (half life of 1.3 days) during first and second responses. These data are obviously similar to our results during the primary hemolysin response for both intact and splenectomized rabbits, as shown in figure 8 (see also Taliaferro, 1955 and 1956). Limited direct evidence is also shown by our finding (W. H. and L. G. Taliaferro, 1957) that a single dose of S^{35}-labeled amino acids given when different amounts of antibody are being formed results in approximately the same specific radioactivity of the antibody (fig. 12). Such a similarity would not occur if stored unlabeled antibody was being released unless it was released at a constant rate over the period studied.

When antibody formation occurs in granulomata as after the use of Freund's adjuvant, antibody may be stored. Hence, the above relationships would not hold. Even after the intravenous injection of antigen, however, Cheng and Haurowitz (1957) believe that antibodies are formed in large amounts during a primary response and are later released after the initiation of the secondary response.

In summary, the induction of antibody formation (= preinduction period) at the beginning of the immunological induction period may involve some reaction between antigen and nucleotides although little is known about the process. Most of the so-called immunological induction period is concerned with the development of the antibody-forming mechanism. Once operative, this mechanism synthesizes antibody directly from the amino acids of the amino acid pool without the intervention of

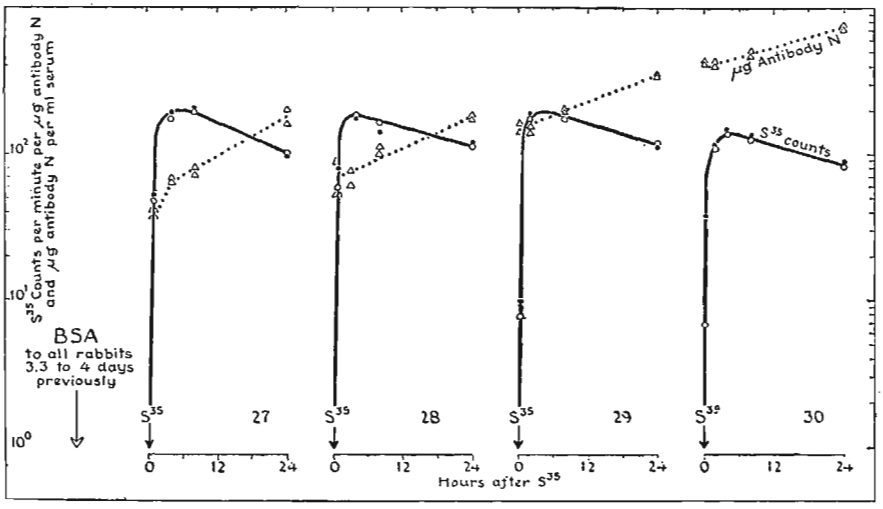


FIG. 12. Data from 4 of the rabbits in figure 11 showing the rise and fall in uptake of S^{35}-labeled amino acids by antibody during the 24-hour interval after one injection of S^{35}-labeled amino acids while precipitins were rapidly increasing in amount. (From W. H. and L. G. Taliaferro, 1957.) Note that the rise and fall in specific radioactivity (solid lines) were more or less uniform although the 4 rabbits were forming different amounts of antibody (dotted lines).

long-lived amino acid precursors. Antibodies are not formed by modifying preexisting proteins. The time of synthesis is less than 40 minutes and may be much less. This short length of time, as ascertained by the intravenous injection of isotopically labeled amino acids, indicates that serum titers under such conditions, suitably corrected for metabolic decay, give a good approximation of the actual rate of synthesis. Antibodies of the same immunological specificity made in the same animal may differ widely in their physico-chemical characteristics, their rate of metabolic decay, their avidity, their primary union with antigen and their ability to trigger and support the secondary immunological reactions. Such diversity makes it difficult to characterize antibody and impossible to measure total antibody in the serum although excellent relative measures are available.

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